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Role of NS1 in virus replication using Dengue virus and West Nile virus chimeras

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ROLE OF NS1 IN VIRUS REPLICATION USING DENGUE VIRUS AND WEST NILE VIRUS CHIMERAS

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For the Glory of Our God

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LIST OF ABBREVIATIONS

DENV	Dengue virus
WNV	West Nile virus
YFV	Yellow fever virus
SINV	Sindvis virus
DHF/DSS	Dengue hemorrhagic fever / dengue shock syndrome
ER	Endoplasmic reticulum
prM	Pre-membrane protein
M	Membrane protein
C	Capsid
NS	Nonstructural protein
RC	Replication complex
MEM	Minimum essential media
FBS	Fetal bovine serum
cDNA	Complementary deoxyribonucleic acid
MOI	Multiplicity of infection
IFA	Immunofluorescence assay

GFP	Green fluorescence protein
Kb	Kilobases
DMEM	Dulbecco's minimal essential media
BHK	Baby hamster kidney
HEK	Human embryo kidney
PBS	Phosphate-buffered saline
BME	β-mercaptoethanol
SDS	Sodium dodecyl sulfate
EDTA	Ethylenediaminetetraacetic acid
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
DAPI	4`,6-diamidino-2-phenylindole
dsRNA	Double strand ribonucleic acid
KDa	Kilodalton
PFU	Plaque forming unit
UV light	Ultraviolet light
SDM	Site directed mutagenesis
DENV/WNV	NS1 from WNV in a DENV genetic background
WNV/DENV	NS1 from DENV in a WNV genetic background

ABSTRACT

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The flavivirus non-structural protein 1 (NS1) is translocated into the endoplasmic reticulum (ER), glycosylated, and secreted from the infected cell. Among its various functions, a role of NS1 in RNA synthesis has been reported. The NS1 has three domains; "β-roll", "wing" and "β-ladder (spaghetti loop)" domain. The characterization of the three domains of NS1 may help us to understand the multiple functions of NS1 in the context of specific regions of the protein. In this study, we have constructed chimeric Dengue/West Nile (DENV/WNV) viruses, which each contain one of the three domains of NS1 from WNV in a DENV genetic background. The chimeras were characterized with respect to viral RNA synthesis, *trans*-complementation and virion assembly. It was observed that the β-roll chimera was impaired in RNA synthesis, leading to decreased production of viral particles. The reciprocal chimera, WNV/DENV β-roll chimera also showed a reduced level of replication. The spaghetti loop chimera was defective in viral RNA synthesis, and formed small plaques. The wing domain chimera did not show viral RNA synthesis. Remarkably this chimera was rescued by mutations in the wing domain. Collectively, the results indicate that these three domains have a role in viral RNA synthesis. The wing domain is not interchangeable between DENV and WNV for DENV RNA replication, while β-roll and spaghetti loop chimeras could support RNA synthesis.

CHAPTER 1. INTRODUCTION

1.1 Dengue virus

Dengue virus (DENV) represents one of the most prevalent arthropod-borne human pathogens within the genus flavivirus. Dengue infection occurs mainly in tropical and sub-tropical regions, and represents an important public health threat. DENV infection causes febrile illness known as dengue fever (DF) or develops into severe disease with hemostasis and vascular permeability referred to as dengue hemorrhagic fever / dengue shock syndrome (DHF/DSS). There is no clinically approved vaccine or antiviral therapy beyond treatment of symptoms. There are four serotypes (DENV-1,-2,-3 and -4), among them DENV 2 is the most prevalent serotype (Hoang et al. 2010). The genome of DENV is a positive-sense, single-strand RNA of approximately 11 kb in length. During infection, the viral RNA is translated into a polyprotein, then cleaved by a cellular or viral proteases into three structural proteins (capsid (C), pre-membrane / membrane (prM/M) and envelope (E)) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins. The structural proteins are components of the extracellular virus particle. The major function of the non-structural proteins (NS) is viral RNA replication. The NS5 protein plays a role as an RNA-dependent RNA polymerase and methyltransferase, and DENV NS3 is RNA helicase and protease. The viral proteins, viral RNA and host cell factors form the replication complex (Puig-Basagoiti et al.) to promote efficient viral replication (Miller and Krijnse-Locker 2008; Salonen, Ahola, and Kaariainen 2005). The pathogenic mechanism and interactions between DENV and host factors are not revealed yet.

1.2 Non-structural protein 1 review

1.2.1 Introduction

The NS1 is a multifunctional 43-48 kDa protein, depending on its glycosylation status. The mutant for the loss of N-linked glycans in N130Q and N207Q showed lower molecular weight than WT NS1 (Somnuke et al. 2011). After translation NS1 is translocated to the ER lumen (Falgout, Chanock, and Lai 1989). In the ER, it is cleaved by unknown host signal proteins, and promptly dimerizes after post-translational modification (Zhou et al. 2006). Then the dimerized NS1 stays in lumen of ER for viral replication, or some NS1 are transported to the cell surface where they remain membrane associated and are released into the extracellular space in hexameric form (Flamand et al. 1999; Crooks et al. 1994). The NS1 that stays in the cell localizes with other non-structural proteins, which are involved in viral replication. It is unclear how NS1 is expressed on the plasma membrane of infected cells. Soluble hexameric NS1 also binds to the plasma membrane of uninfected cells (Alcon-LePoder et al. 2005) via interaction with sulfated glycosaminoglycan (Avirutnan et al. 2007). The hexameric form of NS1 is only found in mammalian cells (Muller and Young 2013). The high level of secreted NS1 in the serum of DENV infected patients is up to 50ug/ml (Alcon et al. 2002) and it is correlated with severe disease. The level of NS1 from DHF patient sera have been shown to be higher when compared with dengue fever patients (Libraty et al. 2002). The NS1 secretion rate is different between DENV and WNV. DENV has higher levels of NS1 secretion than WNV, where WNV accumulates at higher levels on the cell surface relative to DENV (Youn et al. 2010). NS1 facilitates the immune response and has immune evasive functions as it binds to complement proteins and antagonizes their functions (Avirutnan et al. 2010; Avirutnan et al. 2011). In addition to its role in the immune response, NS1 is essential for viral RNA synthesis and replication (Khromykh, Sedlak, and Westaway 1999; Lindenbach and Rice 1997). Interaction with NS4A and NS4B are necessary for successful genome replication (Lindenbach and Rice 1999; Youn et al. 2012). Deletion of NS1 from the viral genome abolishes RNA replication, but this deletion virus can be rescued by *trans* expression of NS1 (Lindenbach and Rice 1997) . Although the role of NS1 in the virus life cycle is well established, the molecular mechanisms of its functions is not known.

1.2.2 Structure of NS1

Understanding the NS1 structure will help to find the molecular mechanism of DENV replication and facilitate vaccine development. The crystal structures for full length NS1 from DENV and WNV are known (Akey et al. 2014). NS1 is stabilized by twelve conserved cysteines which form six disulfide bonds (Blitvich et al. 2001). The NS1 structure comprises three domains (Fig 1.1): a hydrophobic "β-roll" domain (amino acid 1-29), a "wing" (30-180) and a "β-ladder" domain (181-352) (Akey et al. 2014). Shown in Fig 1.1, the NS1 dimer has a central β-sheet domain with a small β-roll domain. The "β-roll" dimerization domain has a structure of two β hairpins, which it is stabilized by a disulfide linkage (Akey et al. 2014). The "wing domain" of each monomer protrudes from the β domain like a wing, the individual wing domain has two glycosylation sites in WNV (Asn130 and Asn175) and one for DENV (Asn130). The wing domain has two α-helixes (Akey et al. 2014). The NS1's third domain, the "β-ladder" is a continuous β–sheet. It forms half of NS1 at its C-terminus, and resembles a ladder. It has a notable long "spaghetti loop" (amino acid 219-272) (Akey et al. 2014). One side of the NS1 domain is hydrophobic, due to the protrusion created by β-roll and connector subdomain interaction (Akey et al. 2015). NS1 dimers interact with the ER membrane and NS4A and NS4B as part of the replication complex (Akey et al. 2014).

The NS1 crystal structure shows a distinct segregation of domains. Each domain appears to be related to a specific function. It led us to test whether each domain has a specific role for NS1 function. The characterization of these three domains will be useful for the development of flavivirus vaccine and anti-viral drugs by engineering of the NS1 protein in DENV and WNV to impair its viral RNA replication.

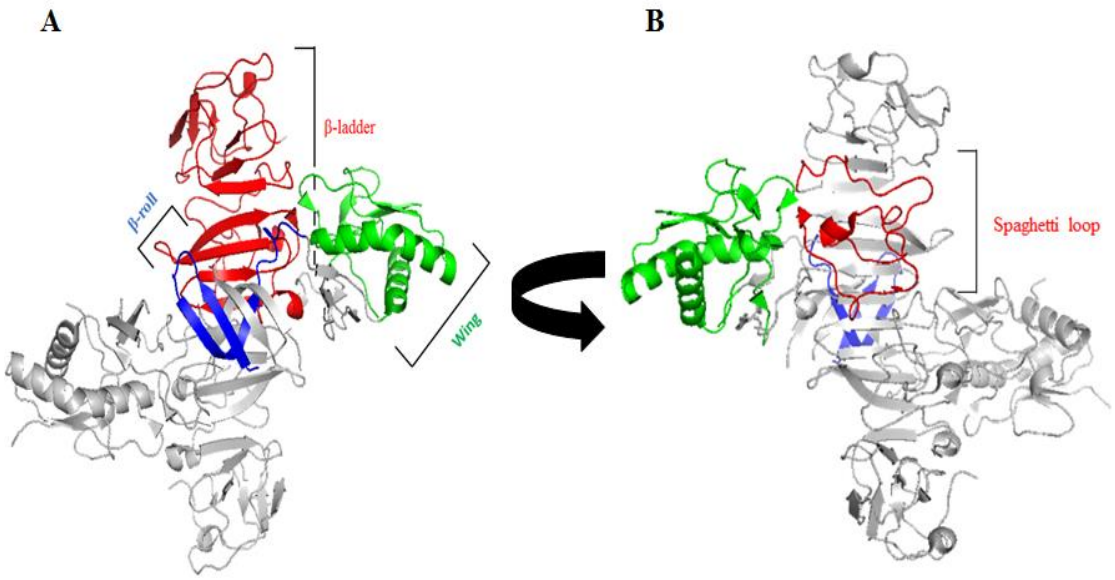

Fig 1.1 NS1 dimer structure. (A) One subunit in NS1 dimer colored in gray and the other colored by domain (blue, β-roll; wing with green subdomain; β-ladder (red) (B) Spaghetti loop in the β-ladder with red. (Akey et al. 2014)

1.2.3 The role of NS1 in virus replication.

Although the exact function of NS1 in viral replication is not known, many studies suggest that NS1 is essential for genome replication (Lindenbach and Rice 1997; Mackenzie, Jones, and Young 1996; Khromykh, Sedlak, and Westaway 2000). After cleavage of the polyprotein, the individual proteins are going to be located on the cytoplasmic or luminal side of the ER. The C protein, NS3 and NS5 are located on the cytoplasmic side, while prM, E and NS1 are on the lumen of the ER. NS2A, NS2B, NS4A and NS4B are in between the lipid bilayer of the ER (Brinton 2014). Although the exact functions of NS2A, NS2B, NS4A and NS4B are not known, it is believed that they interact with one another to form the viral replication complex (Mackenzie and Westaway 2001). NS4B and NS4A have been shown to interact with NS1 (Mackenzie, Jones, and Young 1996; Akey et al. 2014).

Initially it was believed that the role of NS1 was involved in virus assembly (Rice et al. 1986). However pulse-chase experiments found that NS1 remained cell-associated after chase periods. It suggested the cell-associated NS1 localized to sites of RNA

replication and not the site of virus assembly. This result proposed a role in RNA replication as a part of the viral replication complex (Mackenzie, Jones, and Young 1996). To determine its role in viral replication, many mutational experiments for NS1 were performed. Mutations in the NS1 glycosylation sites in yellow fever virus (YFV) showed decreased viral replication levels (Somnuke et al. 2011). Trans-complementation experiment studies reported that NS1 supplied in *trans* could rescue a defective YFV or WNV genome, leading to viral RNA synthesis and replication (Muylaert et al. 1996; Muylaert, Galler, and Rice 1997; Lindenbach and Rice 1997). The trans-complementation of NS1 is species specific, DENV-2 NS1 could not rescue a defective YFV genome (Lindenbach and Rice 1999).

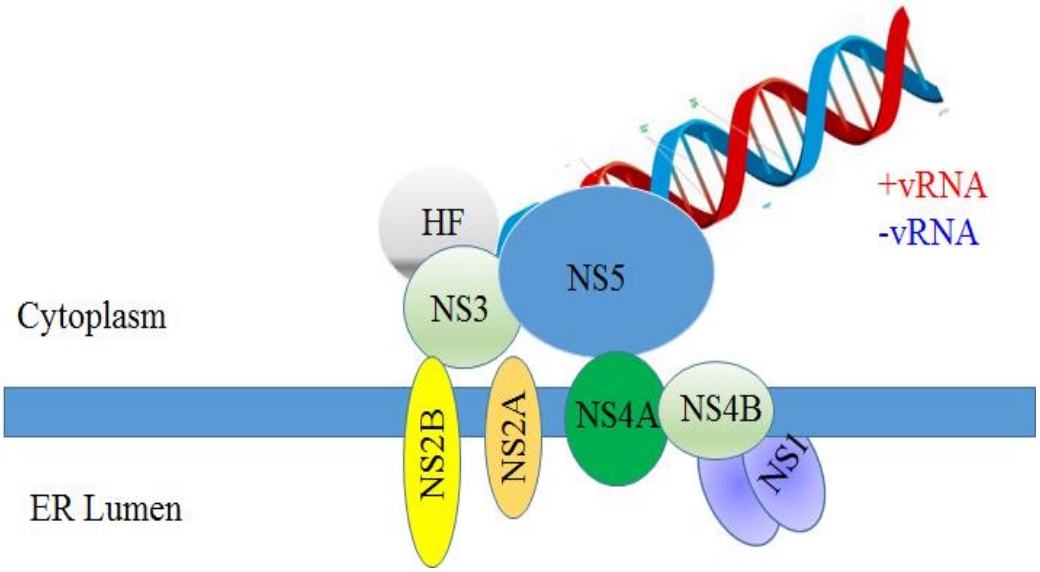

Fig 1.2 Hypothetical model for DENV replication complex. This proposed model came from a consensus in flavivirus replication literature. The replication complex (Puig-Basagoiti et al.) is located in membranous vesicle packets (VP). Seven non-structural proteins and host factor (HF) which are required for replication are RC components. NS1 is located in the lumen of ER (Muller and Young 2013).

Recently it was reported that NS4B interacts with NS1 (Youn et al. 2012). Substitution of the DENV sequence into WNV NS1 counterpart was shown to generate small plaques and reduced viral replication. A variable plaque phenotype emerged within two passages. The rescued mutation was found in NS4B, suggesting that there is an interaction between NS1 and NS4B. Mass spectrometry analysis provided

evidence for a physical interaction between them (Youn et al. 2012). NS1 might be stabilized by physical interactions with regions of NS4B within the lumen of the ER (Muller and Young 2013) (Fig 1.2). However, it is not known whether this interaction is a direct or an indirect one.

1.2.4 The role of NS1 in host immune response

Although NS1 does not exist in the virus particle, antibodies against it are able to protect infection. Immunization with isolated NS1 protects against a lethal challenge dose of virus in mice. (Despres et al. 1991; Jacobs, Stephenson, and Wilkinson 1992). The NS1 of DENV is involved in activating complement, and is associated with increased C5b-9 in DENV infected patient serum (Avirutnan et al. 2006). During dengue infection, NS1 is able to induce humoral immune response. NS1 has been reported to elevate infection in hepatocytes (Alcon-LePoder et al. 2005) and cause immune complex formation (Avirutnan et al. 2006). It also has immune evasion functions such as neutralizing complement activation, resulting in protecting DENV from a direct complement-dependent immune response.

1.2.5 WNV NS1

WNV and DENV are mosquito-borne arboviruses and belong to the genus flavivirus in the family of *Flaviviridae*. WNV causes incidental infections in human with neurological symptoms, such as encephalitis and meningitis (Moreland, Hemmer, and Koht 2014). WNV has an 11 kb positive sense single stranded RNA genome encoding three structural proteins and seven nonstructural proteins as does DENV. WNV has three N-linked glycosylation sites in the NS1 protein at amino acid positions 130, 175 and 207, while DENV contains two glycosylation sites (130 and 207). After being synthesized as a single polyprotein, it is cleaved by host and viral proteases to make individual proteins (Lindenbach and Rice 2003). WNV NS1 is also a multifunctional glycoprotein playing a role in the replication complex (Youn et al. 2012) and is involved in the host immune response (Crook et al. 2014). WNV NS1 is

glycosylated and dimerizes in the ER (Pryor and Wright 1994), then is subsequently secreted from cells in a hexameric form (Chung and Diamond 2008).

CHAPTER 2. MATERIAL AND METHODS

2.1 Cell culture

BHK cells (Baby Hamster Kidney cells) (ATCC, Rockville, MD) were maintained in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). HEK-293T (Human Embryo Kidney 293T) cells were grown in Dulbecco's modification of MEM (DMEM) containing 10% FBS. The cells were grown at 37℃ in the presence of 5% CO_2.

2.2 Construction of Plasmids

A chimeric DENV/WNV virus genome was constructed using the standard overlapping PCR method as described by Yao et al (Yao, Strauss, and Strauss 1996). The Phusion (New England Biolabs, Ipswich, MA) DNA polymerase enzyme was used for all amplifications. pWIIRep-GFPZeo and WNVI-CMVmini-Rep-GFP-AflII were gifts from T. Pierson at the National Institute of Health (NIH). These plasmids contain sequence for the WNV nonstructural proteins and a GFP reporter gene. Each domain from the WNV replicon was introduced into the DENV2 16681 infectious cDNA clone (pD2/IC-MO). The primer sequences are shown in Table 2.1. The β-roll sequences from the WNV replicon were amplified from cDNA pWIIRep-GFPZeo using primers BP1 and CP2. This PCR product was fused to a 5'-terminal DENV-2 fragment amplified from pD2/IC-MO with primers CP1 and BP2. The fusion product was amplified with primers CP1 and CP2, and cloned into *SphI* and *KasI*- cut pD2/IC-MO, yielding the pDENV/WNV β-roll chimera (Fig 2.1). The wing and spaghetti loop chimeras were made by amplifying the domains from pWIIRep-GFPZeo with primers WP2/WP3 or SP2/SP3, respectively. The 5' terminal DENV-2 DNA was amplified with primers CP1/WP1 or CP1/SP1, 3' terminal DENV-2 DNA with WP4/CP2 or SP4/CP2. The PCR product was amplified with primers CP1/WP3 or CP1/SP3. This fragment was fused

again with amplified 3' fragment using CP1/CP2. These final fusion fragments were cloned into *SphI* and *KasI* digested pD2/IC-MO, yielding the pDENV/WNV wing domain chimera or the spaghetti loop chimera. (Fig 2.2) The sequences of all PCR-derived DNA fragments were confiremd by sequencing analysis. The helix substitution plasmid also was constructed by the overlapping PCR method used in chimeric plasmid generation. The loop deletion plasmid constructions were done by the PCR based site-directed mutagenesis (SDM) method. After designing a pair of PCR primers that will delete the loop regions, the entire plasmid is amplified by PCR using Phusion (NEB) DNA polymerase. The template DNA must be degraded by DpnI, which is specific for methylated DNA. The deletion mutant pladmid were left undigested, and were transformed into competent cells.

TABLE 2.1 Primers used in chimera generation. F and R indicate forward and Reverse primer, respectively.

Primer	Location in DENV 2		Location in WNV
CP1 (F)	1380	ACACCATTGTGATAACACCTCACTCAG	N/A
CP2 (R)	3710	AAGTCACGCCCATACCTATGTC	N/A
BP1 (F)	2534	GACACAGGCTGTGCCATTGATATTGGCAGGCAAGAGCTCCGGTGCGGAA GTGGAGTGTTTATCCACAACGATGTGGAAACATGGACAGAACAATAC	1984
BP2 (R)	2429	GGCACAGCCTGTGTCGGCCTGCACCATGACTCCCAAATACAG	1984
WP1 (R)	2530	CTGTGGCGTCTCCGGGTAGAACTTGTATTGTTCTGTCCATGTGTGC	2086
WP2 (F)	2543	GAACAATACAAGTTCTACCCGGAGACGCCACAGGGCCTAG	2086
WP3 (R)	2921	CAAAGCCATAGTCTTCTACCTCCATACTGTTCCATGCTCGG	2424
WP4 (F)	2921	GGAACAGTATGGAGGTAGAAGACTATGGCTTTGGAGTATTC	2432
SP1 (R)	3084	GGTGCATGATTTGACTTCAATGAAAGAGGCTTTCTCTATCTTCC	2638
SP2 (F)	3094	GAAAGCCTCTTTCATTGAAGTCAAATCATGCACCTGGCCAG.	2638
SP3 (R)	3269	GTCCATCTCAAGCTTCCCCTCATCCCATGGGCCTTGGTTC	2772
SP4 (F)	3269	GGCCCATGGGATGAGGGGAAGCTTGAGATGGACTTTGATTTC	2779

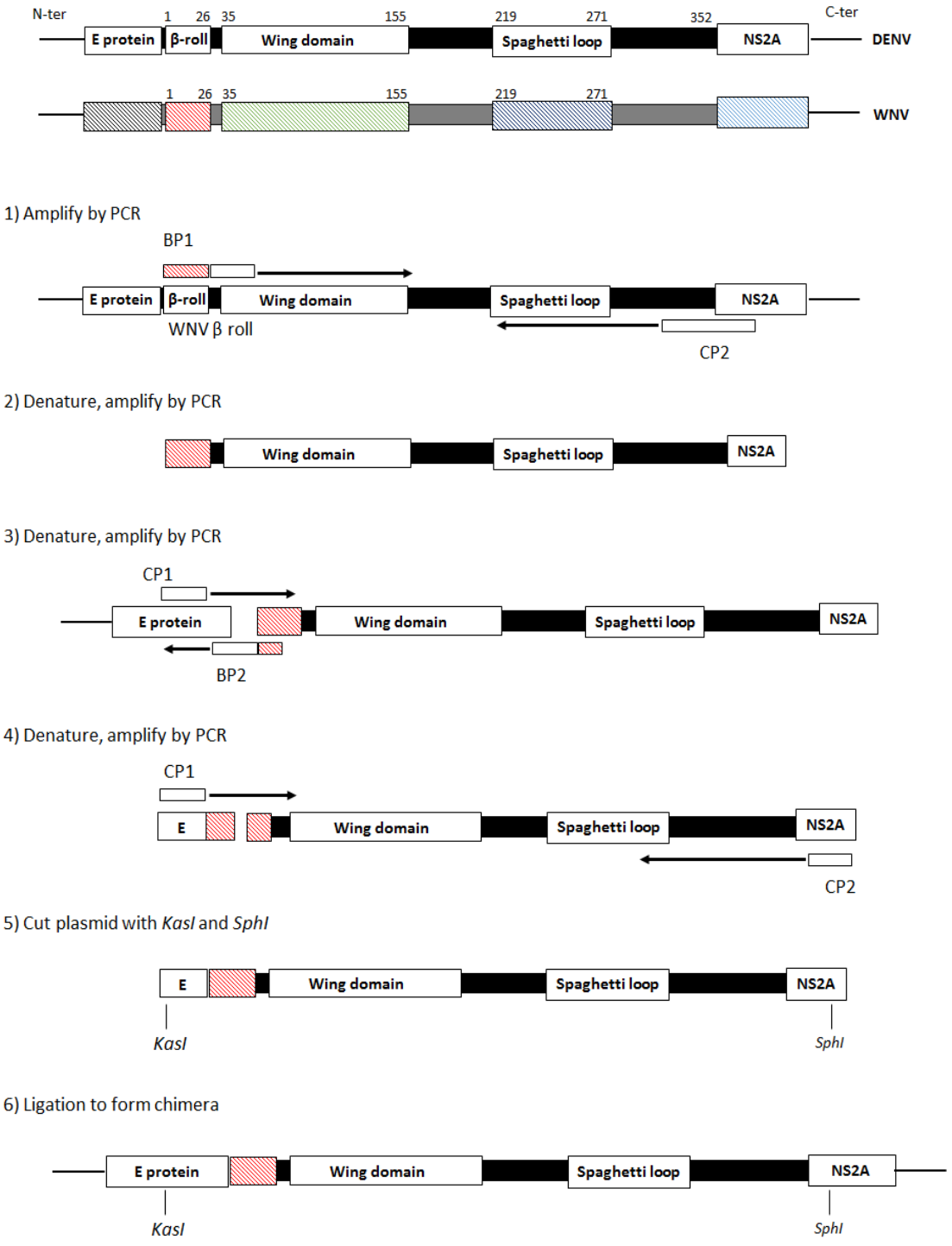

Fig 2.1 Construction of β-roll chimera. In this constructs, the DENV β-roll was replaced with the WNV β-roll

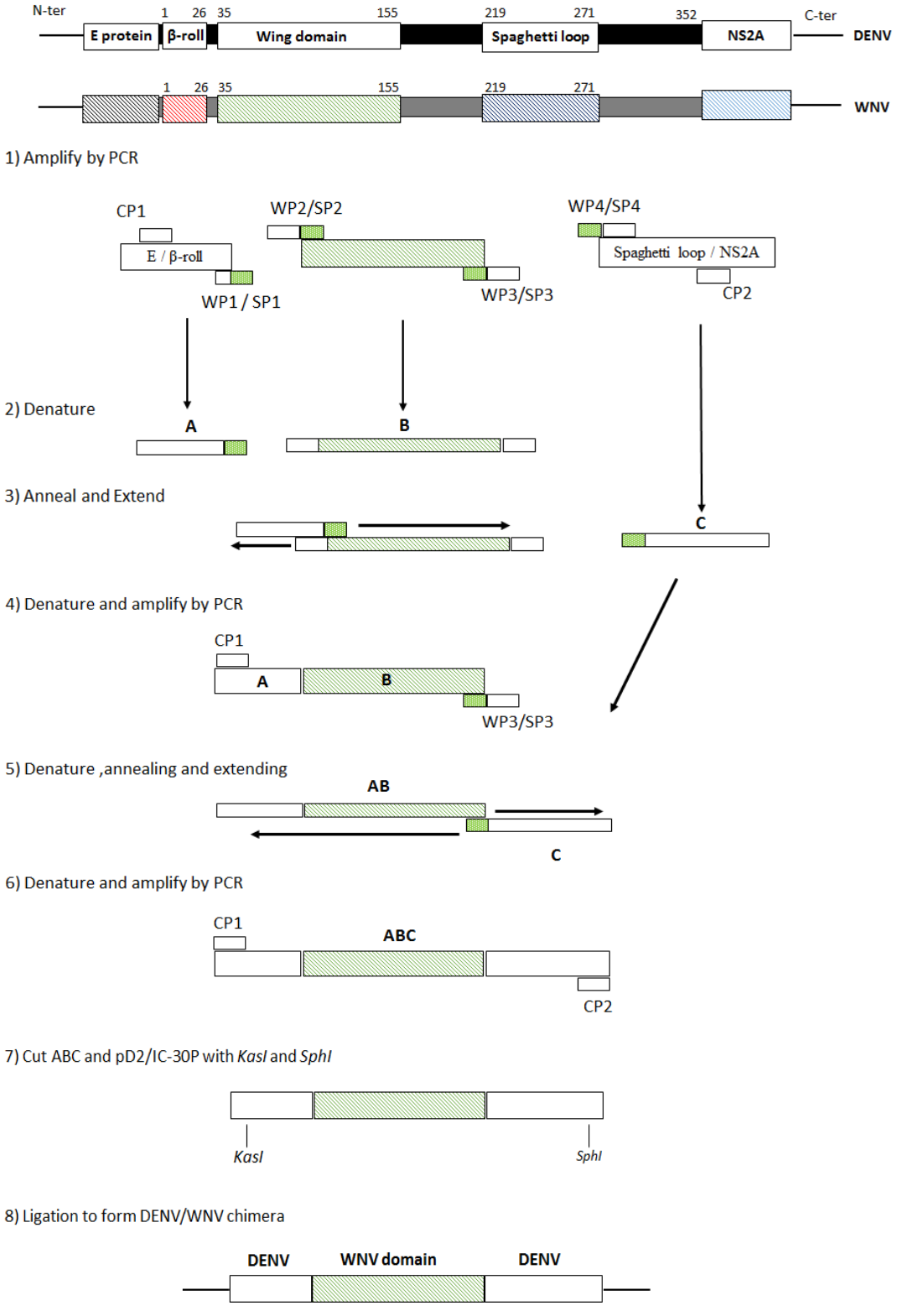

Fig 2.2 Construction of wing domain or spaghetti loop chimeras. Each domain in DENV was replaced by the WNV wing domain or spaghetti loop domain.

2.3 *Trans*-complementation

For the *trans*-complementation experiment, a Kuhn lab member, Michael Dibiasio-White generated an SINV-NS1 construct. Full length Sindbis virus (SINV) cDNA was used as a backbone and DENV (pD2-IC-MO) was used to amplify NS1. The structural proteins were deleted from the SINV clone, leaving the secondary 26S promoter intact. This Sindbis construct was amplified to introduce unique restriction sites for insertion of NS1 (*BsshII* and *Xba1*). Primers were designed and used to amplify NS1 from the DENV cDNA retaining the last 21 amino acids of E protein for correct insertion of NS1 into the membrane. Flanking the 5' & 3' ends of E-NS1 were *BssHII* and *XbaI,* restriction sites, respectively. Vector and insert were then ligated together, and *in vitro* transcription was done to make RNA using an SP6 promoter.

2.4 Linearization and *in vitro* transcription

DENV/WNV chimera cDNA was digested with *Xba1* (NEB). A GFX column (Amersham Biosciences, Piscataway, NJ) was used for purification of the linearized DNA, and *in vitro* transcription was performed using T7 RNA polymerase (Invitrogen, Grand Island, NY) at 37℃ for approximately 2 hours. The presence of synthesized RNA was confirmed by separation on an agarose gel, staining with EtBr (ethidium bromide), and visualization under UV light.

2.5 Transfection of RNA, electroporation.

DENV/WNV chimeric in vitro transcribed RNA was electroporated into confluent BHK cells. The confluent cells grown in T-75 culture flasks were harvested by trypsinization and washed with phosphate-buffered saline (PBS). The collected cells were resuspended in 400μl of PBS, then placed in 2mm gap cuvette (BioRad, Hercules, CA). The *in vitro* transcribed RNA were transferred into the cells in the cuvette. Electroporation was performed with a GenePulser II apparatus (BioRad) with two pulses of 1.5 kV, 25μF and 200Ω. After a 3 min recovery at room temperature, the cells were resuspended in MEM supplemented with 10% FBS and distributed into 6 well lates or 24 well plates or T75 flasks, depending on the experiment.

After electroporation, the integrity of the chimeric RNA in infected cells was investigated. Electroporated cells were washed with PBS at the designated time point, and RNA was extracted from the lysate using an RNeasy kit (Quiagen). Reverse transcription was performed using a specific primer ((+)TAGCCCAGTCAACATAGA AGCAG, (-)AAGATAAGTCACGCCCATACCTATGTC). Reverse transcriptase PCR (Promega Corp, Madison, WI) was used for the amplification of the NS1 region in cDNA. Amplified DNA was purified using GFX columns (Amersham Biosciences), and sequenced to determine the presence of chimera.

2.6 Virus production

After electroporation, cells were resuspended in MEM medium containing 10% FBS and cultured in 24 well plates or T-75 flasks. They were incubated at 37℃ for 5~6 days. On every day from day 1 to 6 post electroporation, 400 μl of supernatant was harvested after centrifugation at 4000 rpm (=3,082xg) for 4 min, and stored at -80℃. Viral titers were determined by plaque assay on BHK cells.

2.7 Plaque assay

BHK cells at ~90% confluence in 6 well plates were washed with PBS. The collected virus was serially diluted in PBS (Ca^{++}, Mg^{++}) and used to infect BHK cells. After 2 hours rocking at room temperature, MEM containing 5% FBS, 1% Agarose, and Penicillin/streptomycin (200 U : 200ug/ml final) covered the cells. The plates were incubated at 37℃ for 5~6 days, and stained using Neutral Red stain (Sigma) for visualization of plaques.

2.8 Immunofluorescence Assay, IFA

BHK cells were transfected with RNA from various chimera constructs using the electroporation method. Immunofluorescence assay (IFA) was performed 1~3 days post electroporation. Cells in 24 well plates were fixed with 80% acetone for 30 min at room temperature. The fixed cells were then washed three times with PBS and incubated for 2 hours rocking with permeabilization buffer (0.5% Triton X and 10% FBS in PBS) at

room temperature. Anti-NS1, -NS5 and -dsRNA antibodies were separately used as primary antibodies, incubated for 1 hour rocking at room temperature. The treated cells were washed with PBS prior to adding secondary antibodies, goat anti-mouse FITC or goat anti-rabbit FITC or goat anti-rabbit TRITC, for 2 hours at room temperature. The antibodies were removed and the cells were stained with DAPI for 5 min at room temperature. The cells were washed with PBS and observed by fluorescence microscopy with a digital CCD camera for image capture (Nikon, Melville, NY). IFA was also used to assess the efficiency of electroporation of BHK cells with *in vitro* transcribed wild type or chimeric RNA.

2.9 qRT-PCR

After electroporation of *in vitro* transcribed RNA into BHK cells, cells were seeded on 24 well plates and cultured for 4 days. On every day from day 1 to 4 post electroporation, the supernatant was collected and the cells were lysed. RNA was extracted from the lysates and supernatants using an RNeasy kit (Quiagen, Valencia, CA) according to the manufacturer's protocol. Extracted RNAs were diluted in 30ul RNase-free distilled water. For real time RT-PCR, 5 µl of RNA elution were used to quantify the RNA copy number using a SuperScript III platinum SYBR green qRT-PCR kit with Rox (Invitrogen). DENV2 specific primers ((+)TTGCGGTGTCAATGGCTAACA, (-) CCAATGCGTTCAATCGGCT) were used for reverse transcription. The quantification was performed using an ABI 7300 thermocycler: 50˚C 4 minutes, 95˚C 5 minutes, and 40 cycles of 95˚C for 5 seconds and 60˚C for 1 minute. Data were collected during the 1 minute 60˚C step. In order to distinguish nonspecific primer binding, we added a single dissociation stage following the run.

2.10 Western blot

After electroporation of BHK cells with wild type or chimeric RNA, cells were cultured for 5 days. Cytoplasmic extracts were obtained after cell lysis in RIPA buffer (50mM Tris-Hcl, 150mM of NaCl, 5mM of EDTA, 1% of NP-40, 0.1% of SDS, 0.5% of Sodium deoxycholate). After centrifugation at 12,000 x g for 20 min, the

supernatants were boiled with 5X sample buffer (0.25M Tris pH 6.8, 10% of SDS, 0.5% of bromophenol blue, 50% of glycerol, 25% β-mercaptoethanol (BME)). Lysates were separated on a 12% acrylamide gel. The proteins were then transferred onto an 0.45μm nitrocellulose membrane at 100V for 1 hour with rocking at ice cold temperature. After blocking overnight in 10 % nonfat dried milk at 4˚C, the membrane was incubated for 2 hours with a monoclonal Ab against NS1. The membrane was further incubated with a secondary antibody against rabbit conjugated with IR680 fluorescent dye for 1 hour at room temperature. Analysis of the blots was performed using an Odyssey scanner (Li-COR bioscience, Lincoln, NE).

2.11 Virus growth kinetics

BHK cells were seeded onto 6 well plates and infected with mock, WT DENV and β-roll chimeric virus at a multiplicity of infection (MOI) of 0.1 for 2 hours at 37℃. Supernatants were collected at every 12 hours and replaced with fresh medium. Virus yields were titrated by plaque assay with BHK cells.

CHAPTER 3. DOMAIN INTERCHANGE BETWEEN DENV AND WNV TO DETERMINE FUNCTION

3.1 Chapter summary

The role of NS1 in RNA synthesis has been previously reported. NS1 is comprised of three domains: the "β-roll", "wing" and "β-ladder (spaghetti loop)" domains. This chapter will delve deeper into the role of each domain in NS1, in particular regarding viral RNA synthesis and virion assembly. We have constructed three chimeric DENV/WNV viruses, which express each domain of NS1 from WNV in a DENV-2 genetic background. This chapter details efforts to characterize the role of the three NS1 domains in terms of replication and virus production. It was observed that the β-roll and spaghetti loop chimeras were impaired in RNA synthesis. Similarly the reciprocal chimera, WNV/DENV β-roll chimera also showed a reduced level of replication. The wing domain chimera did not replicate viral RNA. Remarkably, RNA synthesis of the non-replicating chimera was rescued by *trans* expression of NS1 or mutation of the wing domain chimera with DENV NS1 helix. These results support the hypothesis that each domain has a specific function and certain domains cannot be exchanged between two related flaviviruses.

3.2 Introduction

3.2.1 Construction of chimeric plasmids

The crystal structures for full length NS1 from DENV and WNV have been described (Akey et al. 2014). The NS1 structure comprises three domains (Fig 3.1): hydrophobic "β-roll" domain, "wing domain" and "β-ladder (spaghetti loop)" domain (Akey Brown et al. 2015). Based on this information, three chimeric viruses from a full-length cDNA clone of pD2/IC-MO were generated. The first chimera, called the "β-roll

chimera" consisted of a DENV backbone in which the β-roll domain of DENV was replaced by that of WNV. The second and third chimeras, "wing domain chimera" and "spaghetti loop chimera", were replaced by the corresponding regions from WNV (Fig 3.1).

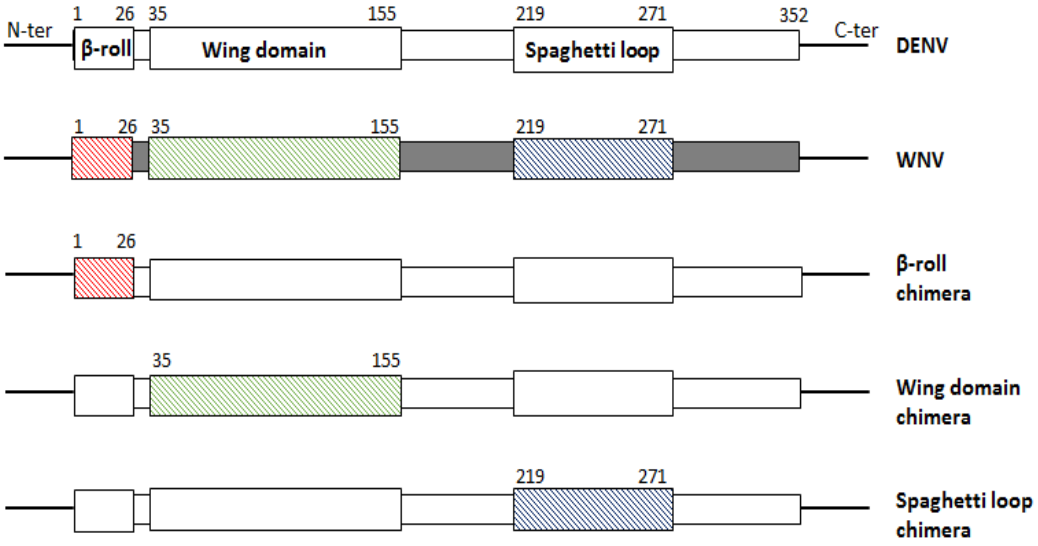

Fig 3.1. Schematic representation of the DENV/WNV chimera constructs. All constructs contained the DENV structural proteins (C, prM and E protein). The WNV β-roll, wing and spaghetti loop domain are indicated by a cross-hatched bar, and DENV-2 gene are indicated by open bars.

3.2.2 The amino acid sequence alignment between DENV2 and WNV

According to the crystal structures of NS1 from WNV and DENV, NS1 showed a similar structure (Akey, Brwon et al. 2014). This observation led us to compare the sequence of NS1 between DENV and WNV. The amino acid sequence of NS1 in DENV was aligned with NS1 in WNV by ExPASy (www.expasy.org). Sequence alignment of NS1 between DENV and WNV revealed 196 identical amino acids. (56 % identity between DENV and WNV) (Fig 3.2). Each domain showed approximately 50% or greater identical residues between two viruses: β-roll (47%), wing domain (50%) and spaghetti loop (66%). The high homology (74%) amino acid sequences of NS1 between DENV and WNV showed.

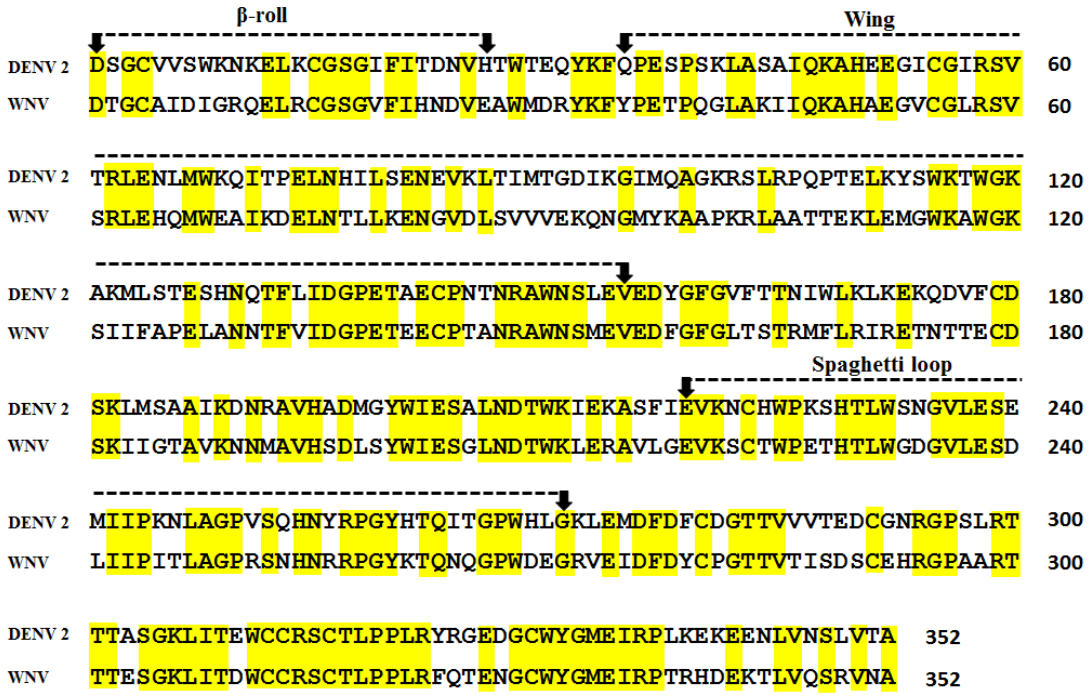

Fig 3.2 Amino acid sequence alignment of NS1 between DENV and WNV. The position of amino acids in NS1 are labeled according to DENV-2 Thailand 16681/84 strain (GeneBank accession number AAB58782), and West Nile virus NY99 (ABA62343). The yellow represents identical residues between DENV2 and WNV.

3.3 Result for IFA and plaque assay

To examine the effect of switching the individual domains on viral RNA replication, we electroporated equal amounts of wild type (WT) and genome-length chimeric RNAs into BHK cells. For the plaque assay, electroporated BHK cells were resuspended in MEM medium containing 10% FBS, and cultured in a T-75 flask. After incubation at 37℃ for 5 days, the supernatant was collected, and clarified by centrifugation at 4000 rpm (=3,082xg) for 5 minute. The supernatant was used to infect fresh BHK cells. In order to test whether the NS1 domain exchange between WNV and DENV caused virus production, and to quantify the DENV virus production, plaque assays were performed. After 2 or 3 days post transfection, cells were examined by IFA using an anti-NS1 antibody.

3.3.1 β-roll chimera

Consistently, β-roll chimera-infected BHK cells showed a distinct NS1 staining in the IFA experiment, compared to mock- infected cells (Fig 3.3A). However, β-roll chimera-infected BHK cells produced fewer NS1-positive cells compared with WT infected cells up to 3 days post electroporation, suggesting a decreased spread of viral infection in this chimera. At 48 hours post transfection, BHK cells infected with the β-roll chimera showed small numbers of NS1 staining. At 72 hours, these cells showed increased NS1 staining, but not as much as WT DENV. This result suggests that substitution of the β-roll domain in NS1 reduced viral RNA replication and protein production.

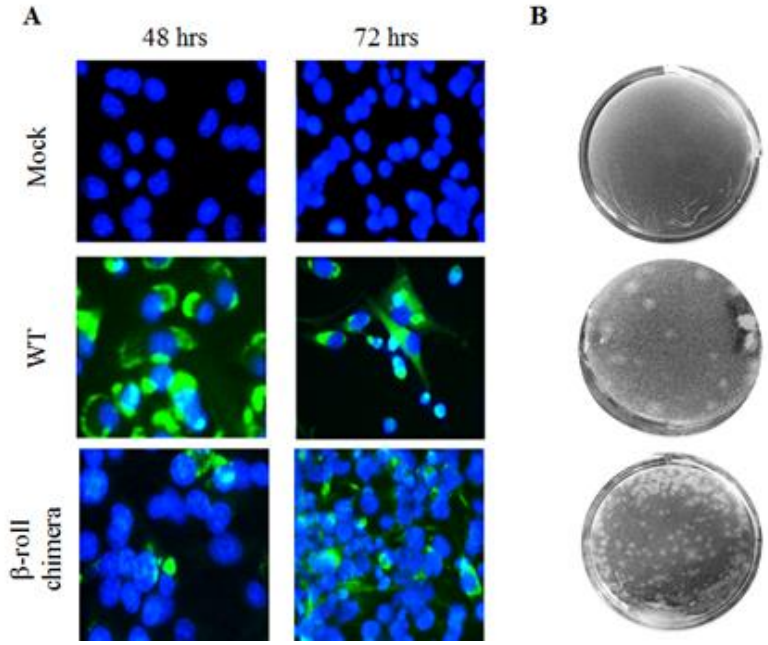

Fig 3.3. Characterization of the β-roll chimera. (A) IFA. BHK cells were electroporated with mock, DENV-2 wild type (WT) or β-roll chimera genome-length RNAs. From day 2 to 3 post transfection, anti-NS1 antibody was used for the detection of replication. The blue and green colors show the nuclei and NS1 protein staining, respectively. (B) Plaque phenotype. Plaques were developed on day 6 post viral infection

A small plaque phenotype was observed for the β-roll chimera, compared to WT infected cells (Fig 3.3B). The BHK cells infected with β-roll chimera produced viral titer that were less than 100-fold lower than those of cells infected with WT DENV. Combined, results from the plaque assay and IFA confirm that the β-roll chimera is

reduced in RNA synthesis and thereby affected in virus production while still able to produce viable plaques. These results demonstrate that the β-roll domain in NS1 might be involved in replication complex formation and may be able to be exchanged between DENV and WNV for DENV viral RNA synthesis.

3.3.2 Spaghetti loop chimera

The spaghetti loop chimeric RNA infected cells were examined by IFA using anti-NS1 antibody. There were a few NS1 positive cells at 48 hours post electroporation. At 72 hours, cell transfected with the spaghetti loop chimera displayed increased NS1 positive cells, although the number of NS1 staining cells were less than WT DENV infected cells (Fig 3.4.A). This result suggested that the spaghetti loop chimera is also reduced in RNA synthesis.

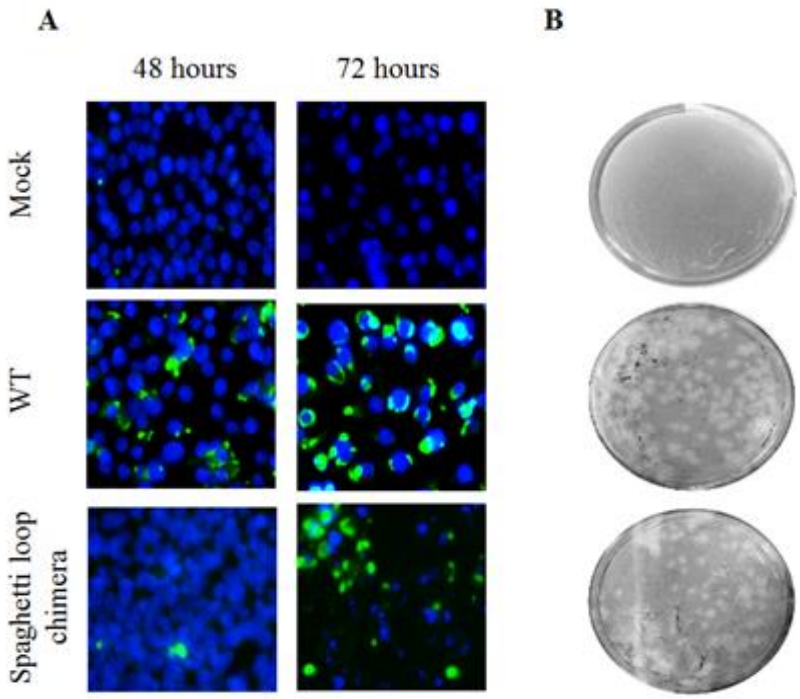

Fig 3.4. Characterization of the spaghetti loop chimera. (A) IFA, *in vitro* transcribed RNAs were electroporated into BHK cells. From day 2 and 3 post transfection, NS1 protein expression was investigated for RNA replication. The blue and green colors show the nuclei and NS1 protein staining, respectively. (B) Plaque morphology. Plaques were developed on day 6 post viral infection.

In order to test whether the spaghetti loop domain exchanging the WNV and DENV sequences affected virus production, plaque assays were performed as described for the β-roll chimera plaque assay. The plaque size of the spaghetti loop chimera is illustrated in Fig 3.4B. The plaque size of this chimera was consistently smaller than what was observed in the WT DENV infection. This plaque assay result supports the IFA result that the spaghetti loop chimera may not replicate or assemble as efficiently as WT DENV, but is able to produce a virus. This suggests that the spaghetti loop, like the β-roll domain may be able to be exchanged between the two viruses.

3.3.3 Wing domain chimera

To determine the effect of switching the wing domain in NS1 on viral RNA replication, IFA was performed with anti-NS1, -NS5 and -dsRNA antibodies. The chimera-infected BHK cells did not show any positive staining up to 3 days post electroporation (Fig 3.5A). After incubation at 37℃ for 5 days, the supernatant was collected, and used to infect fresh BHK cells for a plaque assay. No plaques were observed from the wing domain chimera infected cells (Fig 3.5B). Based on the IFA and plaque assay result, the wing-domain chimera displayed a failure in either translation or RNA synthesis, resulting in a lack of plaque formation. To address which process caused the lack of NS1 visualization, RNA accumulation was subsequently monitored by qRT-PCR.

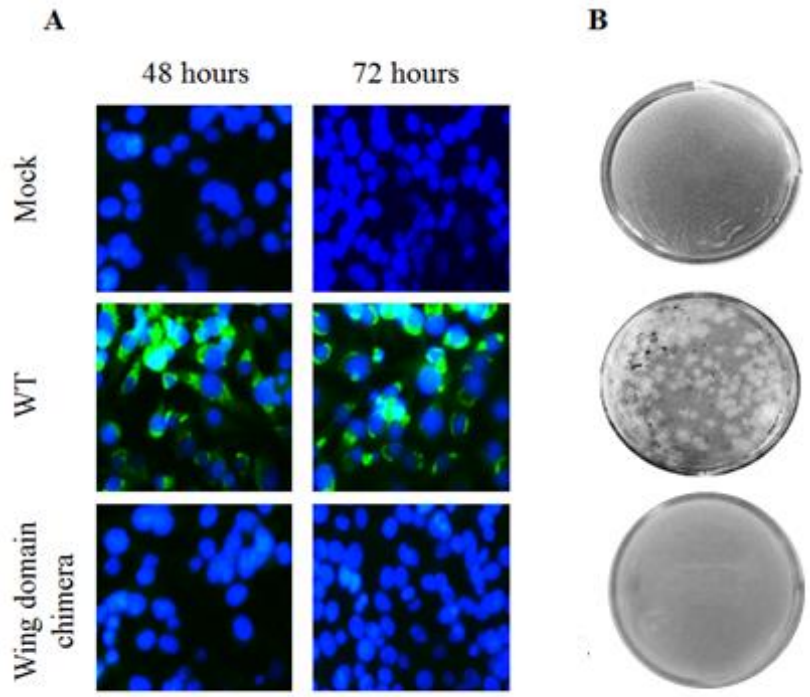

Fig 3.5. Characterization of the wing-domain chimera. (A) IFA. BHK cells were electroporated with mock, WT and the wing domain chimeric RNA. After 2 or 3 days of transfection, the NS1 antibody was used for replication determination. No replication was observed. (B) Plaque assay was performed to check virus production. Wing domain chimera virus could not form plaques.

3.4 Result for Western Blot

In this experiment, we sought to compare the amount of total NS1 produced across the different chimeras. BHK cells were electroporated with mock, WT and three chimeric RNAs, and cells were lysed at 3 days post transfection. Proteins were immunoblotted and probed for NS1. No detectable NS1 proteins were produced from the β-roll chimera infected cells. Western blot analysis showed that the spaghetti loop infected cells were able to produce NS1 at 3 days post transfection (Fig 3.6), supporting the IFA data (Fig 3.4A).

The spaghetti loop chimeric NS1 appeared to express a lower level of NS1 than WT NS1. This is probably as a result of impaired replication. Therefore, the spaghetti loop chimera can replicate to some degree and still make chimeric NS1 but it does not

do so at the same rate as WT NS1. This suggests that the function and residues of the spaghetti loop could be shared between WNV and DENV

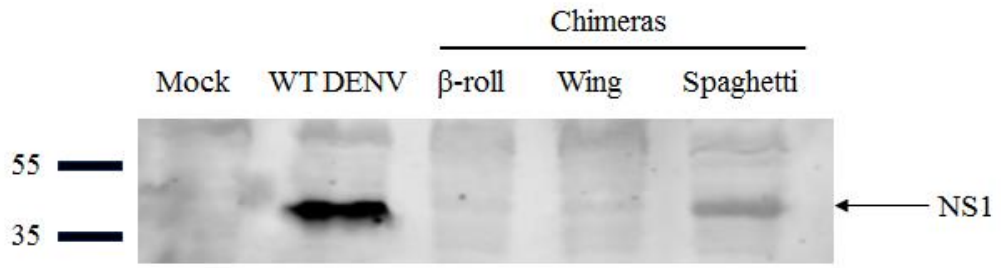

Fig 3.6 Western blot for chimeras. After electroporation of chimeric RNA into BHK cells, lysates were heat denatured, proteins were separated by polyacrylamide gel electrophoresis. Proteins were transferred onto 0.45μm nitrocellulose membrane, and NS1 was detected.

No NS1 protein was observed on the western blot for the wing domain chimera infected lysate (Fig 3.6). The IFA and plaque assay results suggested that this chimera was deficient in RNA replication. However, there may be two interpretations for this result: 1) This chimera cannot synthesize RNA in the infected cells, 2) The chimera is able to synthesize RNA, but there is a translational processing problem that may inhibit protein synthesis. To differentiate between these possibilities, qRT-PCR and *trans*-complementation experiments were employed.

3.5 Result for qRT-PCR

To confirm the IFA, plaque assay and western blot results, qRT-PCR was performed to evaluate viral RNA synthesis. Since RNA copy number correlates with replication, decreased RNA production compared with WT would confirm the delayed replication phenotype.

BHK cells were transfected with mock, WT, β-roll chimeric RNAs, and the negative control, ΔDD which has a deletion of aspartates within the conserved GDD motif in the RNA-dependent polymerase domain. Cells were cultured in 24 well plates for 4 days at 37℃. On every day from day 1 to 4 post electroporation, total RNA was

extracted from cell lysates. The RNAs were quantified on a thermocycler and compared to standards to assess RNA copy number at each time point.

Until 3 days post infection, the viral RNA level from β-roll chimera infected cells were similar with ΔDD, a negative control. Only after 4 days following transfection, β-roll chimera infected cells showed higher RNA levels. Approximately 3 logs reduction in RNA copy number was observed in the β-roll chimera compared with WT (Fig 3.7). This data demonstrated that the β-roll domain might play a role in replication, especially early viral RNA synthesis. Compared with WT, the RNA copy number for the spaghetti loop chimera infected cells was approximately 2 logs reduced at every time point, which showed most active viral RNA replication among three chimeras. Collectively, the qRT-PCR result indicated that the β-roll and spaghetti loop chimeras showed any signs of being able to replicate viral RNA. This result was consistent with the results from IFA, plaque assay and western blot.

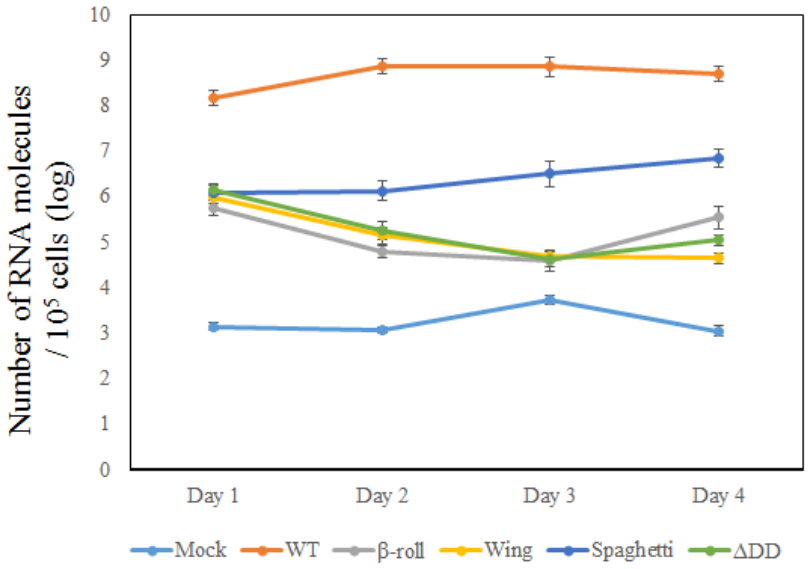

Fig 3.7 Real-time quantitative RT-PCR for chimeras. At every 24 hours after electroporation, total RNA was extracted from lysate and assessed by real-time quantitative. ΔDD was used for negative control

The higher level of RNA molecules than the negarive control, ΔDD means that the β-roll and spaghetti loop chimeras had viral RNA synthesis. But qRT-PCR result for the wing domain chimera showed that there was no detectable RNA replication at any

given time point, suggesting that the wing domain chimera has no viral RNA synthesis. Since the wing domain chimera could not replicate viral RNA, they were not able to translate proteins in this chimera infected cells. This result might explain the data for IFA, plaque assays and western blot in wing domain chimera. Collectively we conclude that the wing domain is required for the DENV viral RNA replication, and it is not interchangeable between DENV and WNV for DENV viral RNA synthesis

3.6 Comparison of growth kinetics of wild type and the β-roll chimera

The growth kinetics of the WT DENV and β-roll chimera were examined in BHK cells. WT DENV and β-roll chimeric viruses were used to infect BHK cells at a MOI of 0.1 for 2 hours at 37℃. Supernatants were collected every 12 hours and replaced with fresh media. Virus yields were titrated by plaque assay on BHK cells. WT DENV virus exhibited a peak titer of ~10^7 pfu/ml at 72 hours post infection. The β-roll chimera showed similar replication kinetics but reached peak titers of ~10^5 pfu/ml at 96 hours after infection (Fig 3.8). The growth curve for the β-roll chimeric virus showed a significant difference from WT DENV from 24 hrs to 72 hrs post infection. The growth kinetics of β-roll chimeric virus were delayed compared to WT DENV.

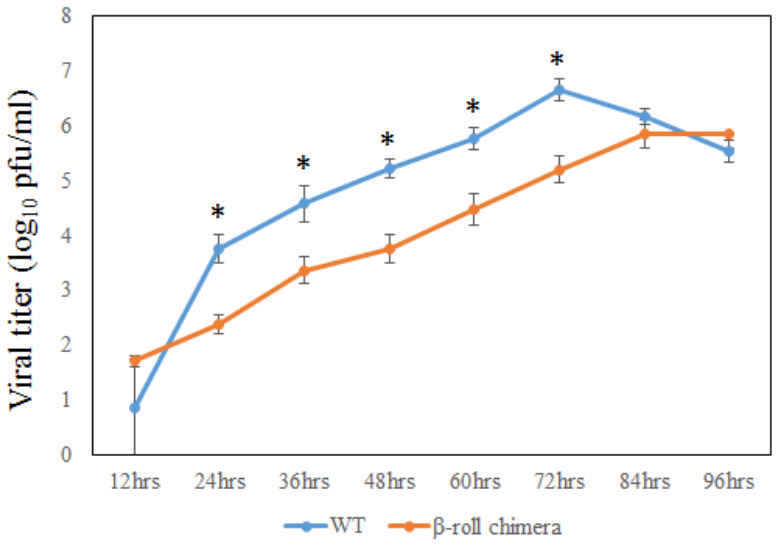

Fig 3.8. Growth curves of β-roll chimeric virus and WT DENV in cell culture. BHK cells were infected at a MOI of 0.1. The virus yield were measured by plaque assay. The viral titers are an average of triplicate samples, and asterisks indicate statistically significant differences (t-test, $p<0.05$).

3.7 β-roll chimera revertants

Although the peak titers of the β-roll chimera were lower than WT DENV, the growth properties of the chimeric virus in BHK cells followed similar kinetics with WT DENV. This gave us the opportunity to analyze the β-roll chimeric virus for revertants which would have allowed the virus to replicate more efficiently in BHK cells. Revertant screens are an important tool to identify critical residues for selected viral proteins. After infection of the β-roll chimeric virus into BHK cells, plaques were selected, and each plaque was passaged as an independent virus. After culturing for 5~6 days, total cellular RNA was extracted, and RT-PCR and cDNA sequencing were used to identify revertants. This screen identified four amino acid changes in NS1 at positions 6, 19, 22 and 26. The residue 26 was changed back to a basic amino acid (Histidine, E26H), as seen in WT DENV NS1, and amino acid at position 6 of NS1 was also changed back to WT (Valine, I6V). Additionally, V19M and H22P substitutions were found from the revertants results (Table 3.1)

TABLE 3.1 β-roll chimera revertant analysis. Amino acid sequence alignment after sequencing of viral RNA from β-roll chimera. 4 amino acids in β-roll chimera were changed. Among them 2 residue were changed back to WT DENV. I (isoleucine), V(valine), M(methionine), H(histidine), P(proline), T(threonine) and E(glutamic acid),

	Residue #6	Residue #19	Residue #22	Residue #26
β-roll chimera	I (ATT)	V (GTG)	H (CAC)	E (GAA)
Revertant	V (GTT)	M (ATG)	P (CCC)	H (CAC)
DENV	V (GTG)	I (ATT)	T (ACA)	H (CAC)

3.8 Generation of WNV/DENV β-roll chimera in a WNV replicon

Collectively, our findings suggest that the β-roll in NS1 is an interchangeable domain between DENV and WNV, despite some impairment in RNA synthesis. This leads us to test whether the reciprocal chimera, WNV/DENV β-roll chimera, is also able to replicate in HEK-293 T cells. The WNV replicon, WNVI-CMVmini-Rep-GFP-AflII were gifts from T. Pierson at the National Institute of Health (NIH). DENV NS1 was from pD2/IC-MO. The strategy for generation of WNV/DENV β-roll chimera was the same as DENV/WNV as described in Material and Methods using the specific primers ((+) GATAGTGGTTGCGTTGTGAGCTGGAAAAACAAAGAACTG AAAT GTGGCAGTGGGATTTTCATCACAGACAACGTGCACGCTTGGATGGACCGGT ACAAGTATTACC, (-) GCTCACAACGCAACCACTATCAGCGTGCACGTTCAC GGAGAGGAAGAGCAG). The efficiency of the WNV/DENV β-roll chimeric plasmid electroporation was very low (<10%) in BHK cells and thus, Lipofectamine 2000 (Invitrogen) was used to transfect the WNV/DENV β-roll chimeric DNA into HEK-293 T cells. HEK-293 T cells were transfected with mock, WT WNV replicon and the WNV/DENV β-roll chimeric plasmids. At 48 hours post infection, the cells were fixed and examined for GFP expression (Fig 3.9). WNV/DENV β-roll chimera infected cells showed GFP expression, indicating replication in the cells. This data confirmed that the β-roll in NS1 is an interchangeable domain between DENV and WNV.

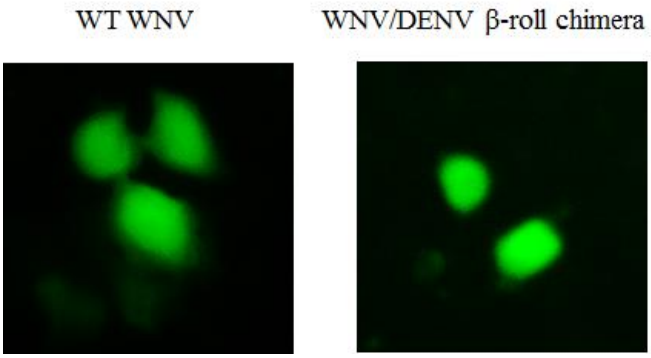

Fig 3.9 Immunofluorescence assay for WNV/DENV β-roll chimera. WNV encoding β-roll chimeric RNAs were electroporated into HEK-293T cells. At about 48 hours post transfection, the GFP expression level was examined for the determination of viral RNA replication.

3.9 The effect of loop deletion in NS1

The spaghetti loop looks like an unstructured region because it lacks secondary structure, yet it is highly ordered by 57 hydrogen bonding (Akey et al. 2015). The spaghetti loop is located on the outer face (Akey et al. 2015). To examine the effect of the loop regions on viral RNA replication, loop deletion mutants were generated by site directed mutagenesis. Fig 3.11 displays the loop amino acid sequence and structure to be deleted. The deleted amino acid at the corresponding positions and primers for mutant generation are shown in Table 3.2. Δ1~3 are located in the spaghetti loop, and downstream of spaghetti loop Δ4 was also investigated. Genome length RNAs containing WT or deletion mutants were electroporated into BHK cells. After 2 or 3 days of transfection, IFA analyses were performed to examine the presence and localization of these mutants using anti-NS1, -NS5 and -dsRNA antibodies. None of the mutants could be detected by IFA (Fig 3.10). *Trans*-complementation experiments using SINV-NS1 were performed to investigate whether the supply of *trans* WT NS1 could rescue these mutants. The expression of NS1 and NS5 were examined from transfected BHK cells by IFA analysis. Because SINV-NS1 transfected cells could not express the NS5 gene, the detection of NS1/NS5 protein double positive cells could suggest that these loop deletion mutants were rescued by trans-supply of NS1.

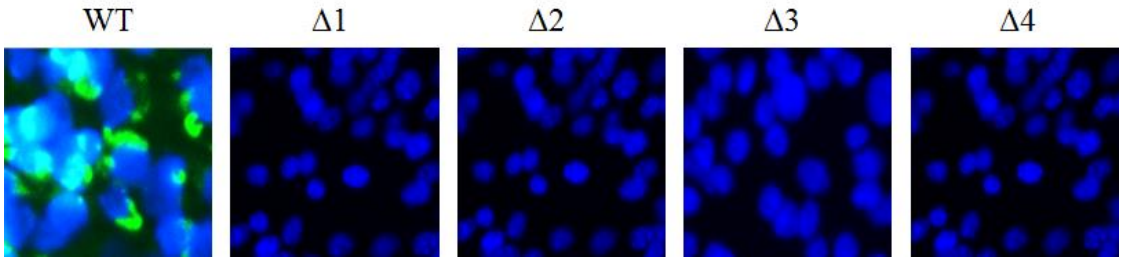

Fig 3.10. Immunofluorescence assay for DENV loop deletion mutant. The loop deletion mutants RNAs were electroporated into BHK cells. 2 days of post transfection, anti-dsRNA antibodies were used for viral RNA replication determination.

TABLE 3.2. The deleted amino acid at the corresponding positions and primers for mutant generation

Deleted loop residue	Primer	
Δ 1 (219-270)	(+)	GAGAAAGCCTCTTTCATTGGTAAGCTTGAGATGGACTTTGAT
	(-)	AATGAAAGAGGCTTTCTCTATC
Δ 2 (223-246)	(+)	CTTTCATTGAAGTTAAAAACCTCGCTGGACCAGTGTCTCAACAC
	(-)	GTTTTTAACTTCAATGAAAGAGGC
Δ 3 (248-270)	(+)	GATAATTCCAAAGAATCTCGGTAAGCTTGAGATGGACTTTGATTTC
	(-)	GAGATTCTTTGGAATTATCATCTC
Δ 4 (303-306)	(+)	CCCTCTTTGAGAACAACCACTCTCATAACAGAATGGTGCTGCCGATC
	(-)	AGTGGTTGTTCTCAAAGAGGGTCC

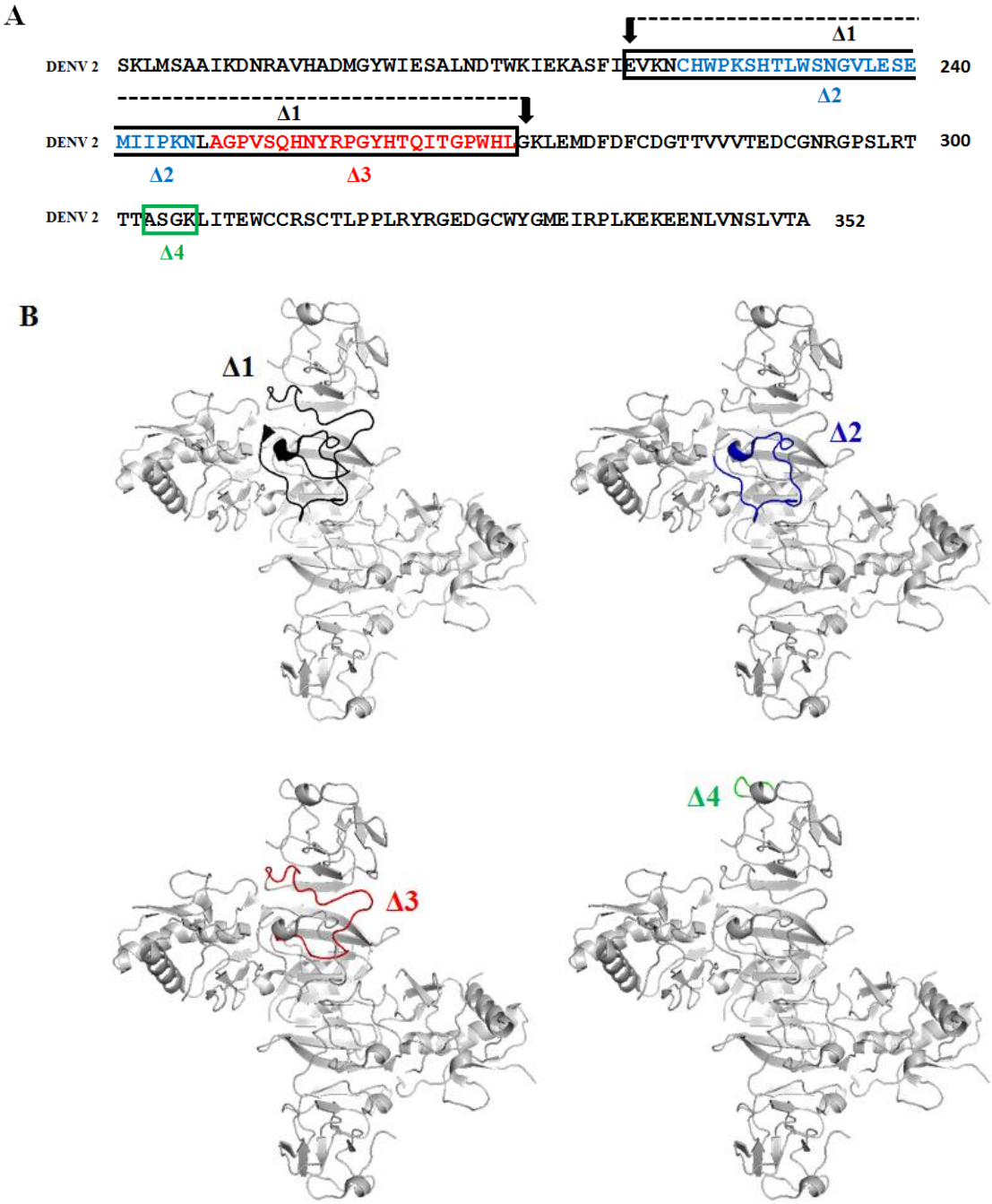

Fig 3.11. Generation of loop deletion mutants. (A) Amino acid sequence for the deleted loop regions. The dotted line represents the spaghetti loop. Black and green boxes indicate Δ1 and Δ4 deletion mutant, respectively. Blue and red, Δ2 and Δ3, respectively. (B) Deleted loop regions in DENV NS1 structure.

Among four loop deletion mutants, only Δ4 mutant could be rescued by *trans*-complementation (Fig 3.12). The loop deletion in the spaghetti loop region abolished RNA synthesis, suggesting that the loop is necessary for RNA replication.

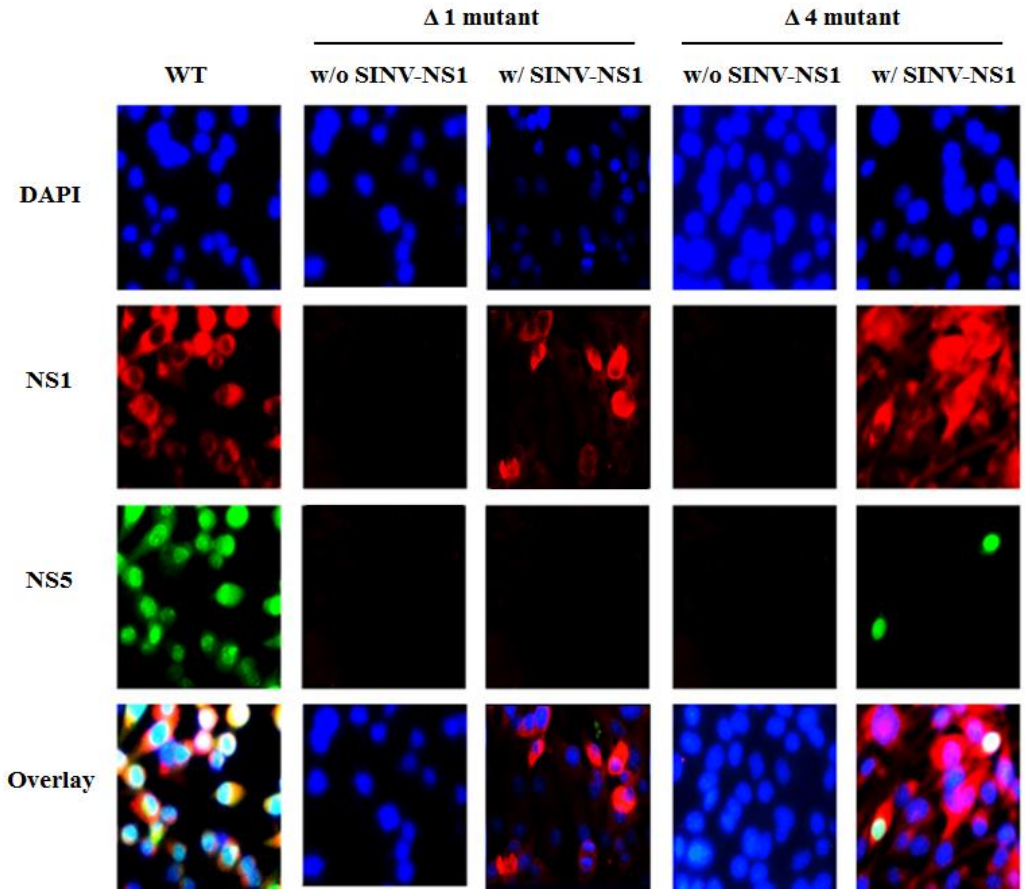

Fig 3.12. IFA for *trans*-complementation with loop deletion mutants. Δ 1~4 mutant RNAs were electroporated into BHK cells in the presence or absence of SINV-NS1. After 2 or 3 days post transfection, IFA analysis was performed to examine viral RNA replication. The results for Δ2 and Δ3 are same with Δ1.

3.10 *Trans*-complementation for the wing domain chimera using SINV-NS1

The defect in viral RNA synthesis and virion assembly in the wing domain chimera had led us to attempt the *trans*-complementation assay with SINV-NS1. Genome- length RNAs containing the wing domain chimera were electroporated into BHK cells with SINV-NS1 RNAs. The transfected BHK cells were examined for NS1 and NS5 expression with IFA. Because SINV-NS1 does not have an NS5 gene, the detection of NS5 protein-positive cells could demonstrate the replication of wing-

domain chimeric virus RNA. After electroporation of the chimeric RNA with the SINV-NS1 RNA, functional NS1 was provided in *trans* to the non-replicative wing domain chimera. NS5 positive cells were clearly observed in the BHK cells. But neither NS1 nor NS5 positive signals were detected in the wing domain chimeric RNA transfected cells without SINV-NS1, showing the wing chimeric RNAs are non-replicative (Fig 3.13). This data demonstrated that the wing domain chimera was able to translate proteins NS1 and NS5, and the RNA synthesis is lethal without a WT NS1 protein present.

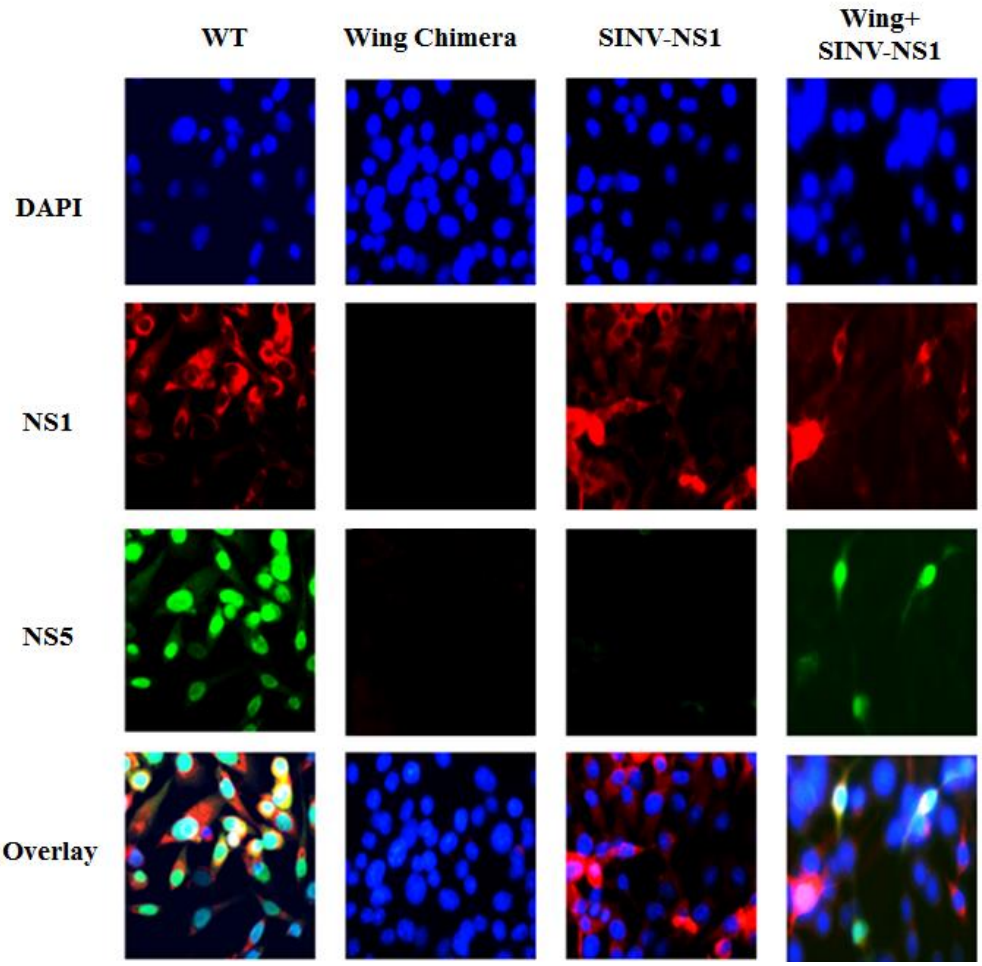

Fig 3.13. IFA for *trans*-complemtation with wing domain chimera. BHK cells were electroporated with equal amounts of WT or the wing domain chimera genome length RNA in the presence or absence of SINV-NS1. After 2 days post transfection, NS1/ NS5 double positive cells were detected.

3.11 Generation of a helix substituted mutation in the wing domain

In the wing domain of NS1, there are two helices based on the crystal structure of NS1 (Fig 3.14) (Akey et al. 2015). Since these two helices are located proximal to the ER membrane region of NS1, these helices were chosen to be substituted with WT DENV NS1 helix. To investigate whether the non-replicative wing domain could be rescued by replacing it with DENV NS1 helix, site directed mutagenesis was carried out to generate helix substituted mutations on the wing domain chimera. The helix #1 substituted mutant was named as H1, the helix #2 substituted mutant was named H2. Replaced residues in wing domain and the primers for mutant generation are shown in Table 3.3

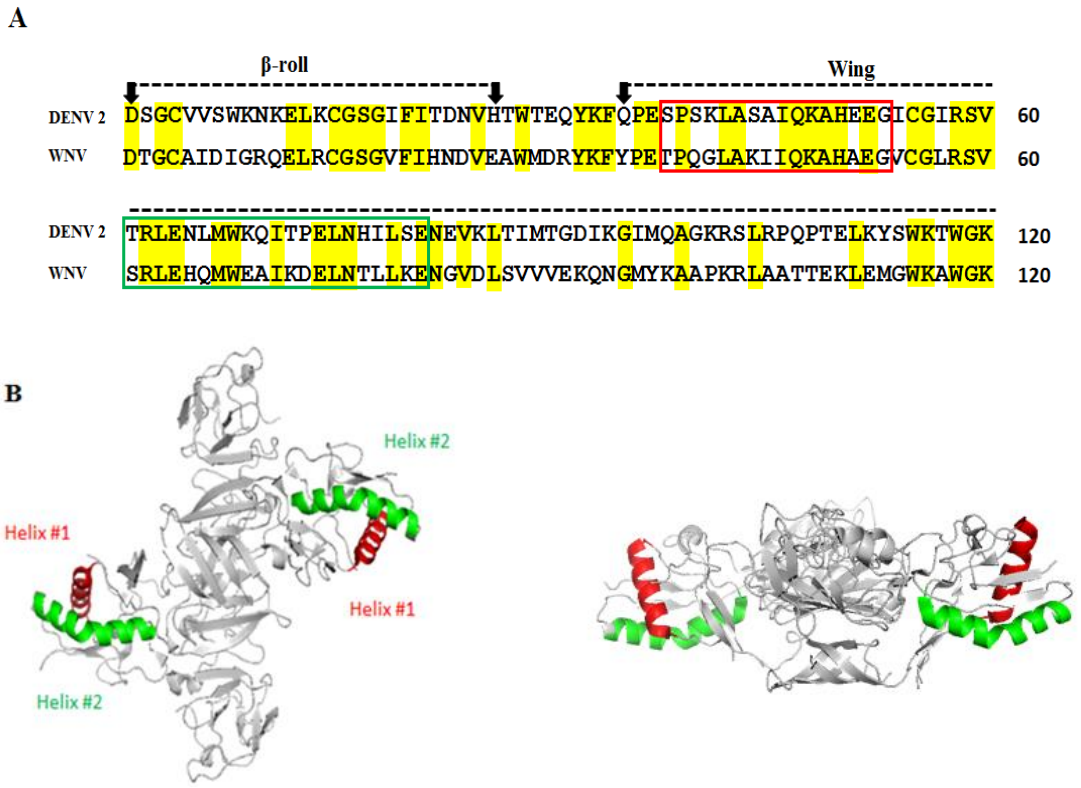

Fig 3.14. Generation of H1 and H2 substitution mutants in the wing domain chimera. Amino acid sequence alignment of the helix in the wing domain between DENV and WNV. The number on right indicates the residue number in NS1. The amino acid at the corresponding positions are indicated. Helix #1 is located at the position from 38-53, and #2 from 61-81. (A). Helix of the wing domain in NS1 structure (helix #1 is shown in red, and green represents helix #2) (B).

Table 3.3. Replaced residues in the wing domain and the primers for mutant generation.

Replaced residue		Primer
Helix #1	(+)	TCCCCTTCAAAACTAGCTTCAGCTATCCAGAAAGCCCATGAAGAGGGCGTCTGCGGCTTGCGTTCCGTTTCC
(38-53)	(-)	CTGAAGCTAGTTTTGAAGGGGAGAACTTGTATTGTTCTGTCCATGTGTGC
Helix #2	(+)	ACAAGACTGGAGAATCTGATGTGGAAACAAATAACACCAGAATTGAATCACATTCTATCAGAAAATGGAGTCGACTTGAGTGTCGTG
(61-81)	(-)	GTTTCCACATCAGATTCTCCAGTCTTGTAACGGAACGCAAGCCGCAGACTCCTTC

To examine the effect of helix substituted mutations in the wing domain chimeras, the *in vitro* transcribed RNAs from WT and each mutated wing domain chimera were electroporated into BHK cells. Every 24 hours after electroporation, the electroporated cells were examined by IFA for viral RNA replication. On the basis of the IFA result, the H1 mutation rescued the non-replicative wing domain chimera (Fig 3.15). This result indicated that the helix #1 is specifically required by DENV, and cannot be substituted by WNV counterpart. The non-replicating wing domain chimera could be rescued by a single helix.

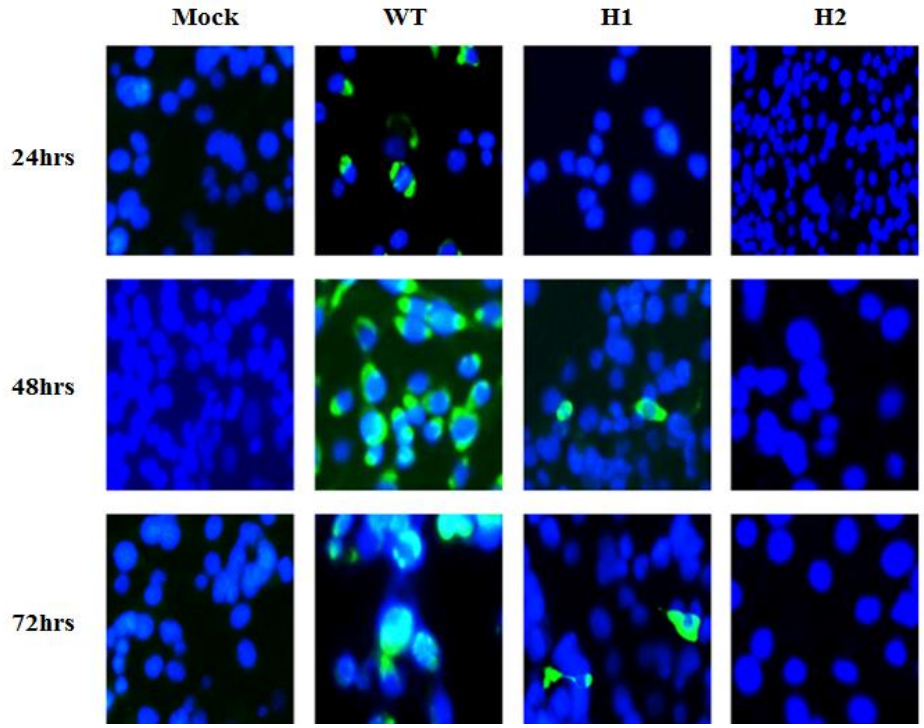

Fig 3.15. IFA for helix substituted mutations in wing domain chimera. BHK cells were electroporated with equal amount of WT or individual helix mutated wing domain chimera. From day 1 to 3 days post transfection, cells were fixed and analyzed for NS1 proteins expression

The *trans*-complementation assay was performed to test whether the failure in RNA replication of helix #2 substituted wing domain chimera could be rescued or not. Genome-length RNAs containing WT and helix #2 substituted wing domain chimera with or without SINV-NS1 were electroporated into BHK cells. On day 2 to 3 post transfection, cells were investigated by IFA for double expression of NS1 and NS5. NS1/NS5 double positive cells were observed from helix #2 substituted wing domain chimera in the presence of SINV-NS1 (Fig 3.16)

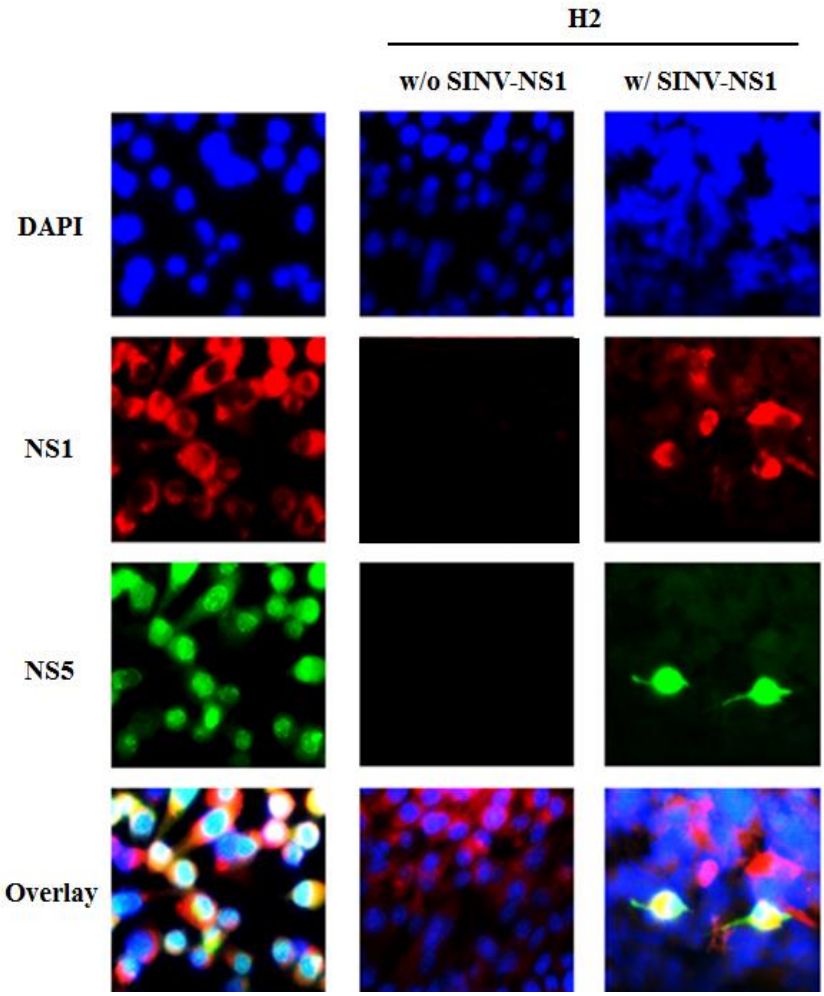

Fig 3.16. IFA for *trans*-complementation assay with H2 mutant. *In vitro* transcribed RNAs from WT, H2 and SINV-NS1 were electroporated into BHK cells. At 2 to 3 days post transfection, IFA assay were performed using anti-NS1 and -NS5 antibodies

3.12 Discussion

Flavivirus NS1 oligomerizes in the ER lumen, and is expressed on the plasma membrane of infected cells. It is also secreted into the extracellular space, the reason for which is unknown. ER luminal localization occurs despite the fact that viral RNA replication occurs on the cytoplasmic part of the ER. Although there are many studies on the role of flavivirus NS1 by several groups, it is not well known what the mechanism is of its participation in viral replication. A previous study has shown that physical interaction exists between NS1 and NS4B, suggesting a mechanism of how ER luminal NS1 affects RNA replication (Youn et al. 2012). It was reported that a lack of NS1 glycosylation might affect the secretion of NS1 from infected cells, resulting in reduction of virus growth (Somnuke et al. 2011). It was also shown that NS1 regulates early viral RNA replication (Somnuke et al. 2011). Collectively, these studies have indicated a role for NS1 in viral RNA synthesis. Previously our laboratory has suggested that the β-roll domain represents a "hydrophobic protrusion" structure that in some way assists replication efficiency. This chapter summarizes the work done on chimeric DENV's carrying mutant NS1 proteins in an attempt to establish the role of each domain in replication and assembly of the virus.

The DENV/WNV β-roll chimera showed impaired RNA replication in the IFA, resulting in decreased production of viral particles in plaque assays. The qRT-PCR data supported this result, showing a 3 log decreased number of RNA molecules in the chimera compared with WT DENV. The reciprocal chimera, the WNV/DENV β-roll chimera also showed a reduced level of replication. This result has several implications. Firstly, it was reported that the rate of NS1 transported from the ER to the extracellular place is negatively related with replication (Youn et al. 2010). If chimeric mutations in the β-roll could cause more rapid transport from ER, it could lead to less NS1 in the ER required for replication resulting in lower infectivity (Youn et al. 2010). To support this hypothesis, further investigation is required using pulse-chase studies. Secondly, the β-roll chimera might alter the destination of chimeric NS1 by affecting its interactions with viral and non-viral host factors. A pull-down assay could be a tool to test specific protein-protein interactions. Thirdly, as the IFA and qRT-PCR data showed, the β-roll chimera might cause delayed replication complex formation, suggesting that some function for early replication complex formation is hindered.

It is interesting that the DENV/WNV β-roll chimera did not produce a second site mutation in NS4B. It was reported that using the WNV encoding an RQ10NK mutant produced a suppressor mutation (F86C) in NS4B after serial passage (Youn et al. 2012). This data led us to look for a second site mutation. However, a second site mutation was not found in NS4B (data not shown). Instead of second site mutations in NS4B, the β-roll chimera appeared to mutate its own sequence such that it became more similar to WT DENV NS1. Four amino acid changes were discovered at position 6, 19, 22 and 26. Positions 6 and 26 reverted back to WT DENV NS1 amino acids. The amino acid at position 6 of NS1 is exposed to the inner face and located near the residue 10 and 11, which were involved NS4B interaction (Youn et al. 2012). The changes at amino acid positions 19 and 22 were not found in other flaviviruses: DENV1, 3, 4, YFV, JEV (Japanese encephalitis virus), SLEV (St. Louis encephalitis virus), MVEV (Murray valley encephalitis virus) and TBEV (Tick-borne encephalitis virus). Position 26 amino acid (His, CAC) changed from (Glu, GAA) showed same nucleotide with WT DENV (His, CAC). This substitution appears likely that this potential revertant might contaminate with WT DENV. We are still in the process of identification for the revertant.

There are some reports that the amino acids 320 (proline) and 333 (methionine) of NS1 have an effect on antiviral signaling (Lazear et al. 2013; Blitvich et al. 2001). Despite these reports, the precise role of the β-ladder (spaghetti loop) domain during infection is not well defined. IFA, plaque assay, qRT-PCR and western blot data showed that the spaghetti loop chimeric RNA-infected BHK cells reduced viral RNA synthesis, but were able to maintain viral infection with the chimeric protein. We speculate that the reasons why impairment occurred might be that the spaghetti loop might affect the NS1 protein folding or the interaction with other non-structural proteins to form the replication complex. Protein misfolding could be tested by using conformational specific monoclonal antibodies, recognizing specific conformational state of NS1. A pull-down assay with chimeric RNA infection could be used for confirming the protein-protein interaction. Compared with other chimeras, however, the spaghetti loop chimera was the most active in viral RNA synthesis as measured by qRT-PCR. This suggests that the spaghetti loop interaction with other proteins may not be affected as much as other chimeras. The small size of the plaques observed with the spaghetti loop chimera

support the hypothesis that the spaghetti loop chimeric virus does not replicate or assemble as efficiently as WT DENV. It was interesting finding that the viral titer for spaghetti loop chimeras (10^3 pfu/ml) was lower than the β-roll chimera (10^5 pfu/ml), although RNA copy number for the spaghetti loop chimera infected cells showed 10-fold greater than β-roll chimera infected cells. This result suggests that the spaghetti loop might be associated with DENV assembly. All of the generated loop deletion mutants including the spaghetti loop resulted in a lethal replication phenotype, confirming that these loop regions might be involved in DENV replication. *Trans*-complementation analysis displayed that the spaghetti loop deletions could not be rescued by supplying NS1 in *trans*. These results have two implications. (i) The spaghetti loop might be involved in replication complex formation, so a functional spaghetti loop must be supplied in *cis* in order for replication to proceed efficiently. (ii) In order to initiate viral RNA replication, it is necessary to form replication complex. If the deletion mutant abolish the translational process for the initial replication complex formation, these deletion mutant could not synthesize any viral RNA.

In DENV RNA replication, the spaghetti loop domain might engage in viral / host protein-protein interactions to regulate viral RNA synthesis. Based on plaque assays and qRT-PCR results, we speculate the spaghetti loop might be involved in DENV assembly. The exact role of the spaghetti loop in NS1 is still an object of study, and these studies describe an unknown role of this loop for viral RNA replication or assembly.

Before showing the crystal structure for full length flavivirus NS1, there were some reports that investigated the role of the wing domain regions (Blitvich et al. 2001; Somnuke et al. 2011; Fan, Liu, and Yuan 2014). They examined the N-linked glycosylation at position 130, because glycosylation of NS1 is essential for polymerization in the ER. They suggested that N130 is required for stabilization of a secreted hexamer form of NS1 (Somnuke et al. 2011). Some groups researched the highly conserved cysteine (Cys) residues which are necessary to form disulfide bonds (Blitvich et al. 2001). They found that the mutant at Cys position 55 impaired RNA synthesis, and consequently failed to form plaques (Fan, Liu, and Yuan 2014). However, the glycosylation site and cysteine residues were not changed in the wing domain chimera, because DENV and WNV have identical residues at these positions. Even

though these glycosylation state and cysteine resides were not changed, RNA replication was abolished, leading to a failure to form plaques. These results suggest that besides the glycosylation and cysteine residue activity, the wing domain might be involved in viral RNA synthesis through an unknown mechanism. The wing domain could affect the oligomerization status or NS1 interaction with NS4B or other proteins.

The wing domain chimera did not show any viral RNA synthesis after wing domain chimeric RNA electroporation. This means that the wing domain is not interchangeable between DENV and WNV for viral RNA replication, and this domain is required for DENV replication. But this replication-defective chimera was *trans*-complemented, displaying a level of replication activity higher than the mock but less than the wild type level of replication. This result demonstrated that the wing domain chimera did not abolish the initial translational process for replication complex formation.

The wing domain displays two helical structures: residue #1, 38-53 and residue #2, 61-81. Many studies have shown that the helical domains of the DENV envelope protein (E) and precursor membrane (prM) protein have important roles in assembly, protein cleavage and entry (Hsieh et al. 2011; Lin et al. 2011; Hsieh et al. 2014). These data had led us to attempt to exchange the helix of the wing domain between the wing domain chimera and WT DENV. Only the H1 mutant showed viral RNA replication, suggesting that helix #1 might play a role in RNA replication. The dead phenotype could be rescued by a single helix. The helix #1 has 16 amino acids, and 10 amino acids are identical between DENV and WNV. The six residues that are different are located on the outside of the helix. These exposed six residues could be essential for replication complex formation. A potential next step is to identify the essential residue for RNA replication in helix #1 by using mutagenesis.

In summary, each of the domains in DENV NS1 were tested using chimeric plasmid generation. Although it is clear that each domain plays a role in viral RNA replication, further studies for assembly and trafficking with these domains needs to be done.

CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTION

The DENV NS1 structure comprises three domains : β-roll, wing and β-ladder (spaghtetti loop) domains. This work was carried out based on the hypothesis that given the similar structures of NS1 between DENV and WNV, substitution of each domain between viruses may elucidate important domain or residues for the function of the proteins. To address this hypothesis, chimeric NS1 viruses (DENV/WNV) were generated in which each domain was replaced by its counterpart in WNV. This is the first study to characterize the role of DENV NS1 domains individually. We found that the β-roll and spaghetti loop chimeras showed impaired RNA synthesis, and resulted in a smaller size of plaque phenotype compared to the WT DENV. The reciprocal chimera, WNV/DENV β-roll chimera, also showed a reduced level of replication. The wing domain chimera was unable to replicate viral RNA and hence produced no plaques. These observations were verified by qRT-PCR and western blot. The qRT-PCR result displayed that the wing domain chimera was ablated in viral RNA synthesis. Complementation analysis showed that the non-replicative wing domain chimera could be rescued by the trans-supply of WT DENV NS1. These results support that each domain has a role in viral RNA replication.

4.1 Understanding the role of chimeric revertant

Based on the result that the β-roll chimera had a lower viral titer than that of WT DENV, but continued to grow at similar kinetics, we believed that revertants were responsible. This led us to do a revertant screen with the β-roll chimera. Our potential revertant had two amino acids changed to the WT DENV sequence. However, the characterization of this revertant is not completed yet. The further examination will be required to characterize the β-roll chimera revertants. The engineered β-roll chimera

substitutions based on the revertant screening result could be a tool to characterize the revertants.

No revertant viruses were obtained for the spaghetti loop chimera. Despite repeated attempts, the spaghetti loop chimera did not retain the chimeric viral sequence after passaging the chimeric virus. The passaged spaghetti loop chimeric virus had WT sequence not the chimeric sequence. After electroporation of spaghetti loop chimeric RNA into BHK cell, the media were collected for virus post 6 days transfection. The spaghetti loop chimeric virus were cultured on BHK cells for 6 days, and monitored for improved viral RNA replication. After one round of passaging, the spaghetti loop chimeric virus produced small plaques, and an individual plaque was isolated to generate virus. Sequencing of the virus showed that these potential revertant viruses were WT DENV. Even though the plaque size was very small compared with the WT DENV plaque, the virus from this plaque was WT DENV. Further studies are necessary to fully understand this abnormality.

4.2 Comparison of reciprocal chimeric viruses

West Nile virus is another mosquito-transmitted flavivirus belonging to the *Flaviviridae* family similar to DENV. WNV has an 11kb positive sense single strand RNA encoding three structural and seven non-structural proteins. The NS1 protein in WNV is a multifunctional glycoprotein that is a part of the replication complex (Chu and Westaway 1992; Khromykh et al. 1999; Youn et al. 2012). WNV NS1 is involved in the modulation of the host immune response (Crook et al. 2014). DENV NS1 and WNV NS1 yielded an almost identical hexamer in terms of the crystal structure (Akey et al. 2015). Collectively, WNV NS1 and DENV NS1 are very similar to each other. This study describes how each domain of DENV NS1 has a role in DENV viral RNA replication by using DENV/WNV chimera. It is possible that WNV/DENV chimera display different results in viral RNA replication with DENV/WNV chimera. Additional WNV backbone chimera construct generation was not performed due to the time limitation. Further efforts to characterize the WNV/DENV wing or spaghetti loop chimera would help to understand the molecular mechanism in DENV and WNV viral RNA replication.

4.3 Identification of essential residues in each domain for RNA replication

A series of mutants of NS1 have been extensively studied for characterization of the role of NS1 in RNA replication. The mutation of a cysteine site, N-linked glycosylation sites and N-terminal or C-terminal truncation of NS1 provided the genetic information for viral RNA replication as a role of NS1 (Blitvich et al. 2001, Somnuke et al. 2011). The establishment of a comprehensive genetic map of NS1 identifying residues that are essential for viral RNA replication would be a useful tool for understanding the mechanism of NS1 in viral replication. For instance, the helix substitution experiment in the wing domain chimera revealed that the helix #1 is essential for DENV replication. Among 16 amino acids in helix #1, 10 residues are identical between DENV and WNV. Just 6 amino acids change rescued viral RNA replication (Fig 4.1.A). Guided by the crystal structure of NS1 reported by our group, the 6 residues are located outside of helix #1 (Fig 4.1.B). These exposed six residues could be associated with binding of non-structural proteins such as NS4B. Further studies will be required to gain insight into how helix #1 might influence DENV replication.

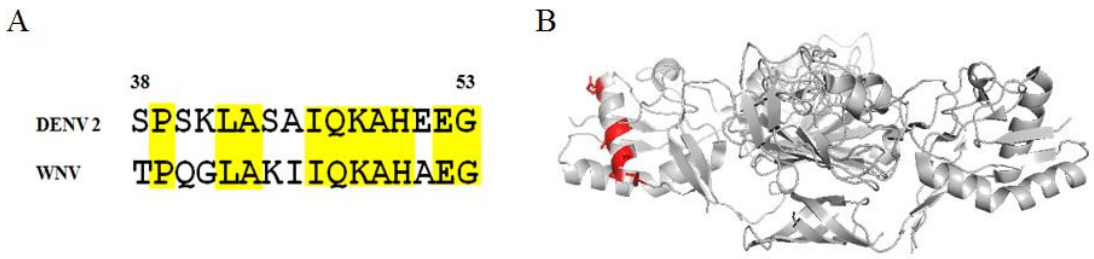

Fig 4.1. The helix #1 sequence alignment and the different residues between DENV and WNV in DENV NS1 structure. (A) The sequence highlighted in yellow comprise a region of identity between DENV and WNV. (B) The different residues between DENV and WNV on helix #1 are indicated in red with side chain.

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